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4. INTRODUCTION

We wish to continue to examine a number of new neuroprotective agents in the MPTP model of PD, which act by inhibiting the mitochondrial permeability transition (MPT) We also wish to utilize metabolomic profiling to identify novel biomarkers for PD and to investigate whether these occur in animal models of PD. We will develop and characterize a new animal model of PD making a knockout of PINK1, which is a nuclear-encoded kinase localized to mitochondria, and which causes autosomal recessive PD. Lastly, we wish to study the effects of human dopaminergic stem cells in the 6-hydroxy-dopamine model of PD.

Outline of Research Goals:

Task 1: To determine the ability of pharmacologic agents to prevent MPTP neurotoxicity (months 1 – 24).

1. Examine neuroprotective effects of phosphodiesterase IV inhibitor rolipram on MPTP.

2. Examine neuroprotective effects of mitochondrial targeted antioxidants (SS02 and SS31), which inhibit the MPT.

3. Examine neuroprotective effects of celastrol and promethazine on MPTP.

4. Examine a novel form of Coenzyme Q_{10} (CoQ_{10}) in the MPTP model of PD.

5. Examine the role of caspase 3 activation in activation of microglia and MPTP toxicity.

Task 2: To develop a new transgenic mouse model of PD by knocking out PINK1, a protein in which mutations cause autosomal recessive PD (months 1-48).

Task 3: To utilize metabolomic profiling to develop biomarkers for PD. We will utilize metabolomic profiling to study patients with PD and animal models of PD (months 1-48).

Task 4. To determine the efficacy of human dopaminergic stem cells in the 6-hydroxydopamine (6-OHDA) model of PD (months 6 -24).

5. BODY

Task 1: To determine the ability of pharmacologic agents to prevent MPTP neurotoxicity.

1. Examine neuroprotective effects of phosphodiesterase inhibitor rolipram on MPTP.

We completed studies with the phosphodiesterase inhibitor rolipram. As reported in our last annual report, we saw protective effects with either 1.2 or 2.5 mg/kg. We saw significant protective effects against dopamine depletion and loss of tyrosine hydroxylase neurons. These results have been prepared for publication.

2. Examine neuroprotective effects of mitochondrial targeted antioxidants (SSO2 and SS31), which inhibit the MPT.

We have made substantial progress in these studies. SS02 and SS31 are part of a recently developed group of small peptides. They all contain alternating aromatic and basic amino acid residues with dimethyltyrosine providing ROS scavenging properties. Initial *in vitro* assay show that SS02 and SS31, but not SS20 dose dependently inhibited lipid peroxidation in scavenged H^2O^2 as measured by chemoluminescence. Uptake studies of radioactive SS31 showed that it concentrates approximately 5,000 fold in the mitochondrial pellet. SS31 decreases mitochondrial ROS production and inhibits MPT and swelling. It also prevents cytochrome c release induced by calcium and inhibits 3-nitropropionic acid (3-NP) induced activation of the MPT, as well as its toxicity in cell cultures.

We, therefore, tested the ability of these compounds to protect against MPP+ toxicity in cell culture and against MPTP toxicity in mice. We found that *in vitro* both compounds decreased mitochondrial ROS production. They inhibited MPP+ induced mitochondrial swelling and attenuated MPP+ induced inhibition of oxygen consumption in isolated mitochondria. They also significantly protected against MPP+ induced apoptotic cell death.

We examined the effect of SS31 in mice treated with an intensive MPTP regimen (10 mg/kg) given as four doses every two hours. Dopamine levels in the striatum were depleted by 84% by MPTP, but were significantly protected against by SS31 treatment. DOPAC and HVA levels showed similar protective effects of SS31. We also examined the effects of SS31 in mice treated with a milder MPTP regimen. This was 10 mg/kg given as three doses every two hours. Dopamine depletion in the striatum by MPTP alone was 44%. SS31 showed significant dose-dependent protection against MPTP induced dopamine depletion. Similarly, DOPAC and HVA levels showed the same dose dependent protective effects of SS31. It restored the levels back to those seen in controls in these experiments. We also observed significant protection against loss of tyrosine hydroxylase neurons in the substantia nigra pars compacta. These results are presently

being analyzed further and we expect to write a manuscript describing these results within the next 6 months.

3. Examine neuroprotective effects of celastol and promethazine on MPTP.

We completed studies of both promethazine and celastrol during our last report. We found marked neuroprotective effects of both compounds. Both of these results have been reported in the *Journal of Neurochemistry* and in *Neurobiology of Disease* respectively.

4. Examine a novel form of coenzyme Q10 (CoQ10) in the MPTP model of PD.

We carried out a large number of studies examining the effects of both oxidized, as well as reduced CoQ10 in the MPTP model. We also examined the effects of a special emulsion, as well as a super emulsion formulation. We initially examined the four different preparations by gavage. We found a marked dopamine depletion of 88%, yet there was still protection by both ubiquinone and ubiquinol. The protection was slightly better with the ubiquinol. There were increased cortical concentrations of CoQ10, as well as marked increases in the plasma. We subsequently repeated these studies and again found that there was protection and that this was somewhat better with the ubiquinol than with ubiquinone. We also examined the dose dependency of neuroprotective effects when administered in the food. We found a significant protection with 0.8% in the food. There was approximately a 50% protection, which was accompanied by a marked increase in plasma CoQ10 levels. The lower doses of 0.1, 0.2 and 0.4% in the food were ineffective. We also examined a number of different concentrations of Tishcon CoQ10 in the food. This preparation is said to result in improved plasma concentrations. We found significant protection at 0.8%. This was approximately a 50% protection. There were marked increases of plasma concentration of about 32-fold with 0.8% in the diet. These studies, therefore, show for the first time that administration of CoQ10 in young mice does exert significant protective effects against MPTP toxicity. Furthermore the reduced form (ubiquinol) is somewhat more effective than the oxidized form ubiquinone.

The analysis of these studies is ongoing. We hope to prepare a manuscript in the coming year.

5. Examine the role of caspase 3 activation in activation microglia and MPTP toxicity. We have continued our studies of matrix metalloprotease3 (MMP3) and its role as a novel signaling proteinase from apoptotic neuronal cell that activates microglia. This is of importance since microglia can utilize NADPH oxidase to generate superoxide. We found that matrix MMP3 was newly induced and activated in stressed dopamine cells. The active form of MMP3 was released into the medium Activated MMP3 as well as catalytically active recombinant MMP3 (CMMP3) led to microglial activation and superoxide generation in microglia. It enhanced dopamine cell death.

CMMP3 caused dopamine cell death in mesencenphalic neuron glia mixed culture of wildtype mice, which was attenuated in a culture of mice, which had a deficiency of NADPH oxidase. These were specifically a subunit null mice known as GP91phox-1. This suggested NADPH oxidase mediated the CMMP3 induced microglial production of superoxide dopamine cell death. Furthermore, in MPTP injected MMP3 deficient mice nigrostriatal neuronal degeneration, microglial activation and superoxide generation were markedly attenuated. These results indicate that active recombinant MMP3 released from stressed dopamine neurons is responsible for microglial activation of NADPH oxidase to generate superoxide. They suggest that this enhances nigrostriatal dopamine neuronal degeneration. These results point to a novel therapeutic target for PD.

We have extended these studies. Exposure of mesencenphalic neurons, tetrapterin, (BH4) an obligatory cofactor for dopamine synthesis results in apoptosis of dopaminergic neurons. We found that the BH4 exposed apoptotic dopaminergic cells resulted in expression of MMP3. This was induced during apoptosis by formation of its active form. No other subtypes of MMPs were induced or activated in response to BH4 exposure. Several serine protease inhibitors attenuated the formation of activated MMP3. Both NNGH and doxycycline, which lead to lowering of MMP3 activity, attenuated the cell death and activation of capsase 3. SP6 OO125, an inhibitor of JNK, lowered the MMP3 induction. These results further reinforce the observations that activated MMP3 plays an intracellular role in neuronal degeneration as a microglial activated molecule.

Task 2: To develop a new transgenic mouse model of PD by knocking out PINK1, a protein in which mutations cause autosomal recessive PD (months 1 -48).

We have generated the PINK1 knockout mice. Correctly targeted ES cells were used for injection to generate the PINK1 knockout mouse founders. We are now expanding the colony. We used a combination of PCR and sequencing to confirm the correct genetic manipulation. We are in the process of attempting to obtain PINK1 antibodies to confirm a loss of protein in the mice. We have carried out initial studies to determine whether there is altered phosphorylation of particular proteins in these mice. We have observed a number of different phosphorylated proteins on two-dimensional gels. These studies are continuing. We are also about to carry out detailed functional studies of mitochondria isolated from the PINK1 deficient mice. We are carrying out initial behavioral studies. We have indeed observed a small deficit on the rotarod. Our colony, thus far, is approximately 6 months of age. We intend to continue to breed these mice for further studies and to characterize their phenotype. It will probably take approximately two years to get the initial characterization of these mice completed.

Task 3: To utilize metabolomic profiling to develop biomarkers for PD.

We are utilizing metabolomic profiling to study patients with PD and animal models of PD. Over the past year, we have made considerable progress in this task. We have continued to analyze and acquire samples from control subjects and PD subjects. We are continuing to analyze these samples to improve our numbers and our ability to acquire

metabolomic signatures of PD. We had initially found a unique set of molecules that distinguish PD from control subjects and other CNS disorders. We obtained samples of PD subjects with LRRK2 mutations, (which causes autosomal dominant inherited PD) as well as appropriate controls from our collaborator Dr. Jan Aasly in Norway. We obtained 11 samples from patients with the G2019S mutation in LRRK2. The patients all manifested PD. We also obtained 15 samples from patients with idiopathic PD. We obtained 14 normal controls and 11 patients from families of the G2019S patients who did not have the mutation. Our studies have demonstrated that the controls and the patients who do not have the G2019S mutations yet are family members show complete overlap. The patients with the G2019S mutation can be completely separated from these two groups of controls. Furthermore, using appropriate markers, patients with the G2019S mutation can be separated from patients with the idiopathic PD. We are presently analyzing the G2019S samples to determine which peaks give the best separation. We have also in the past year developed mass spectroscopy. This is now available to us. We hope to utilize this new technology to get more specific structural information about peaks which we have identified which separate PD patients either with the LRRK2 mutation or without any known genetic mutations from controls.

Task 4: To determine the efficacy of human dopaminergic stem cells in the 6-hydroxy-dopamine (6-OHDA) model of PD.

With regard to this task, we have largely completed it. We have obtained very exciting results. We have also carried out in collaboration with Dr. Steve Goldman and Dr. Neeta Roy, a detailed study of the effects of human ES-derived dopaminergic neurons in a rat model of PD. The acquisition of highly-enriched dopaminergic populations is an important prerequisite to using HES derived dopaminergic neurons for cell based therapy. Non-dopaminergic neuronal derivatives may yield unpredictable or deleterious neurologic side effects. Furthermore, incompletely differentiated HES cells are potentially tumorigenic upon implantation. We, therefore, utilized a new strategy for improving the efficiency of dopaminergic neurogenesis from human ES cultures. This involved co-culture with telomerase immortalized human fetal mesencephalic astrocytes during induction of a dopaminergic phenotype using sonic hedgehog and FGF8. Using this means, we achieved a high efficiency enrichment of dopaminergic neurons. This was true in numbers and apparent uniformity compatible with therapeutic implantation. We then assessed the functional utility of these enriched dopaminergic pools by transplanting these cells into 6-hydroxydopamine lesioned-adult rat brain. We found that transplantation of the HES-derived dopaminergic cells mediated a significant, substantial and long lasting restitution of motor function. Specifically, they reversed apomorphine, induced rotations, which are characteristic of unilateral extrapyramidal dysfunction. This improved response to apomorphine, which lasted for the entire ten weeks of behavioral assessment. We also tested measures of motor asymmetry. Using the adjusting step test to transplanted rat performed equally with either paw while the non-transplanted rats used the contralateral paw more than the ipsilateral paw. The total number of steps taken with both paws did not differ between the two groups suggesting a similar level of motor activity. The full improvements occurred by six weeks after transplantation.

We assessed the engraftment of HES-derived dopaminergic neurons as tyrosine hydroxylase positive neurons in the lesioned striata. This was done by examining sections to evaluate the extent of mediolateral spread of xenografted cells. The grafted cells were disbursed over an average radius of 1.6 +/- 0.6 mm. The number of engrafted neurons was on average 140,000. Efficient generation of TH positive neurons was observed on all six animals. The dopaminergic phenotype was maintained in graft border regions.

We then sought to define the cellular phenotypes within the non-dopaminergic central cores of the xenographs. There was no evidence of teratoma or overt anaplasia seen in any of the sections sampled in the six H9 and three H1 engrafted brains, each of which was sampled 70 days after transplantation. However, some mitotic figures were noted in these brains. We then analyzed them for mitosis among the engrafted population, using a number of different approaches. These included histone H3 and PCNA immunolabeling. We also examined BDRU incorporation *in vivo*. This is a marker for mitotic dividing neurons. Approximately 6 ½ % of the neurons showed BRDU incorporation. This suggests a daily mitotic incidence of roughly 4%. Pure cells express histone H3, a maker of M phase while 1 ½ % of the counted cells expressed PCNA, a broader marker of active division. We then immunostained with a panel of undifferentiated HES markers. A large fraction of cells largely localized at the center of each graft, expressed immature neuroproteins including nesting and musashi suggesting that immature neuroepithelial lineage of these cells.

Together, these results suggest the utility of mesencenphalic astroglial coculture in driving dopaminergic neurogenesis from human ES cells, while demonstrating the potential of the result in grafts in mediating behavior recovery in a model of nigrastriatal loss. The findings, however, suggest a need for caution in the clinical application of human ES derived grafts given their potential for phenotypic instability and undifferentiated expansion. These late complications of otherwise successful HES-derived dopaminergic neuronal grafts suggest that strategies not only for high efficiency of dopaminergic neurogenesis but also methods for ridding the preparations of undifferentiated cells need to be developed. This work is being prepared for publication.

KEY RESEARCH ACCOMPLISHMENTS

A. The finding that mitochondrial targeted antioxidants SS02 and SS31 which inhibit the MPT are neuroprotective against MPTP toxicity in mice, as well as against MPP+ toxicity in cell culture.

B. The finding that a reduced novel form of CoQ10 is effective in the MPTP model of PD. We have also demonstrated that there is dose-responsive protection with other formulations of CoQ10 against MPTP toxicity in young mice. We have evaluated the role of MMP3 activation by microglia in MPTP toxicity. We found that MPTP toxicity was significantly attenuated in MMP3 deficient mice. Furthermore, that an activated isoform of MMP was released from injured dopaminergic neurons, which led to the subsequent activation of microglia. This was accompanied by an increase in superoxide generation, which was mediated by NADPH oxidase.

C. We have developed a knockout model of PINK1. We have found that these mice do have behavioral alterations and show abnormalities in their phosphoprotein profiles. These mice are undergoing continuing evaluation.

D. We have continued metabolomic profiling and have now made the observation that we can separate patients with the G2019S LRRK2 mutation from both family members, as well as normal controls. We can also separate these patients from those with idiopathic PD.

E. We have developed a novel technique for increasing the induction of a dopaminergic phenotype in human ES derived dopaminergic neurons. We showed that transplantation of these stem cell derived neurons into the 6-hydroxydopamine level of PD was efficacious in reversing the behavioral deficits. We found that there was no frank anaplasia. However, there were continuing dividing neurons in the center of the graft raising the possibility that neoplasia could occur and, therefore, warranting caution in the use of this approach for treating human PD.

REPORTABLE OUTCOMES

Cleren C, Starkov AA, Claingasan NY, Lorenzo BJ, Chen J, Beal MF. Promethazine protect against 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine neurotoxicity. Neurobiol of Dis 2005; Dec;20(3):701-8. Epub 2005 Aug 26.

Kim YS, Choi DH, Block ML, Lorenzl S, Yang L, Kim YJ, Sugama S, Cho BP, Hwang O, Browne SE, Kim SY, Hong J-S, Beal MF, John TH. A pivotal role of matrix metalloproteinase-3 activity in dopaminergic neuronal degeneration via microglial activation. FASEB 2006, in press

CONCLUSIONS

We have made considerable progress in our research goals. We have shown neuroprotective effects of rolipram, tracetyluridine, celastrol, promethazine, oxidized and reduced CoQ10, SS02 and SS31 in the MPTP model of PD. We have further examined the role of MMP3 activation in activation of microglia and in MPTP toxicity. We have found that this plays a key role. We have developed a new transgenic mouse model of PD by knocking out PINK1. These animals are presently undergoing further characterization. We have continued our studies of metabolomic profiling of PD patients, which show that we can identify unique biomarkers in patients with LRRK2 mutations. We have complete studies of transplantation of human ES cell derived dopaminergic neurons into a 6-hydroxydopamine model of PD and have shown successful restitution of behavioral abnormalities.

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None

APPENDICES

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Kim YS, Choi DH, Block ML, Lorenzl S, Yang L, Kim YJ, Sugama S, Cho BP, Hwang O, Browne SE, Kim SY, Hong J-S, Beal MF, John TH. A pivotal role of matrix metalloproteinase-3 activity in dopaminergic neuronal degeneration via microglial activation. FASEB 2006, in press

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Promethazine protects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity

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Promethazine (PMZ) is an FDA-approved antihistaminergic drug that was identified as a potentially neuroprotective compound in the NINDS screening program. PMZ accumulates in brain mitochondria in vivo and inhibits Ca^{2+}-induced mitochondrial permeability transition pore (PTP) in rat liver mitochondria in vitro. We hypothesized that PMZ may have a protective effect in a mitochondrial toxin model of Parkinson's disease (PD). Mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) sustained a significant loss of dopaminergic neurons within the SNpc that was strongly attenuated by PMZ treatment. However, neither striatal MPP^+ concentrations nor MPTP-induced inhibition of mitochondrial complex I were affected by PMZ treatment. In isolated mouse brain mitochondria, PMZ partially prevented and reversed MPP^+-induced depolarization of membrane potential and inhibited the Ca^{2+}-induced PTP in brain mitochondria. The sum of data indicates that PMZ is a strong neuroprotective agent capable of protecting dopaminergic neurons against MPTP toxicity in vivo.

Keywords: Promethazine; MPTP; MPP^+; Parkinson; Neuroprotection; Mitochondria; Permeability transition pore; Membrane potential; Complex I; Mice

Introduction

Parkinson's disease (PD) is a progressive disorder leading to degeneration of a large number of dopaminergic neurons in the substantia nigra pars compacta (SNpc), which results in depletion of dopamine (DA) in the striatum (Hornykiewicz, 1966). The causes of PD remain unidentified but substantial evidence suggests an etiology involving both environmental and genetic factors (Moore et al., 2004; Greenamyre and Hastings, 2004). Administration of the mitochondrial complex I inhibitor MPTP, in primates and rodents, reproduces the characteristic degeneration of nigrostriatal dopaminergic neurons with a depletion of striatal dopamine levels (Heikkila et al., 1984). Both mitochondrial dysfunction and oxidative stress appear to play a role in the pathogenesis of Parkinson's disease (Beal, 2003; Blum et al., 2001).

PMZ is a clinically approved drug that could readily be moved to late-stage preclinical trials and then into clinical trials. It was identified as a neuroprotective compound as part of the NINDS program to screen 1040 FDA-approved compounds in a variety of high-throughput assays (Stavrovskaya et al., 2004). Promethazine protected primary neuronal cultures subjected to oxygen/glucose deprivation and reduced infarct size and neurological impairments in mice subjected to middle cerebral artery occlusion/reperfusion (Stavrovskaya et al., 2004). In experiments with isolated rat liver mitochondria, PMZ delayed the Ca^{2+}-induced mitochondrial permeability transition pore (PTP) without impairing mitochondrial physiology (Stavrovskaya et al., 2004). In brain cells, in vivo, PMZ has been shown to accumulate into mitochondria and vesicles (Muller, 1996). We examine the neuroprotective effects of PMZ treatment in the MPTP model of Parkinson's disease. We found a strong neuroprotective effect and in vitro experiments led us to hypothesize that PMZ may be neuroprotective due to its stabilizing effects on mitochondria.

Material and methods

Reagents

All reagents were purchased from Sigma (St. Louis, USA). Promethazine and MPTP were dissolved in phosphate-buffered saline (PBS).

Animals and procedures

The experiments were carried out on mice, in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All

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procedures were approved by the local Animal Care and Use Committee. Male Swiss Webster mice (12 weeks old, 4 per cage) were maintained in a temperature/humidity-controlled environment under a 12-h light/dark cycle with free access to food and water.

MPTP treatments

Protocol 1

Experiments involving measurements of neurodegeneration (TH, Nissl, DA) required sacrificing mice 7 days after treatment in order to observe the total cell death and to avoid later compensatory effects. A preliminary dose effect showed that an injection of a 5-mg/kg/dose of PMZ, twice a day, was insufficient to protect against MPTP-induced decrease in DA levels, while the 10-, 20-, and 40-mg/kg/dose were protective (Fig. 1A). Consequently, mice (9–12 per group) were injected with the lowest dose of PMZ that showed protection (10 mg/kg ip) or with PBS (10 ml/kg ip) 1 h before and 12 h after the first MPTP injection. Mice were treated with MPTP (10 mg/kg ip q 2 h × 4 doses) according to a standard dosage protocol (Schmidt and Ferger, 2001) or with PBS (10 ml/kg). Mice were sacrificed 7 days later by cervical dislocation.

Protocol 2

In order to determine if PMZ disturbed MPTP metabolism, we examined the time course of changes in MPP^+ striatal concentrations following PMZ treatment. We used the highest concentration of PMZ (40 mg/kg ip) that protected against MPTP-induced DA depletion in our preliminary experiment. Mice (9–12 per group) were injected with PMZ (40 mg/kg ip) 1 h before a

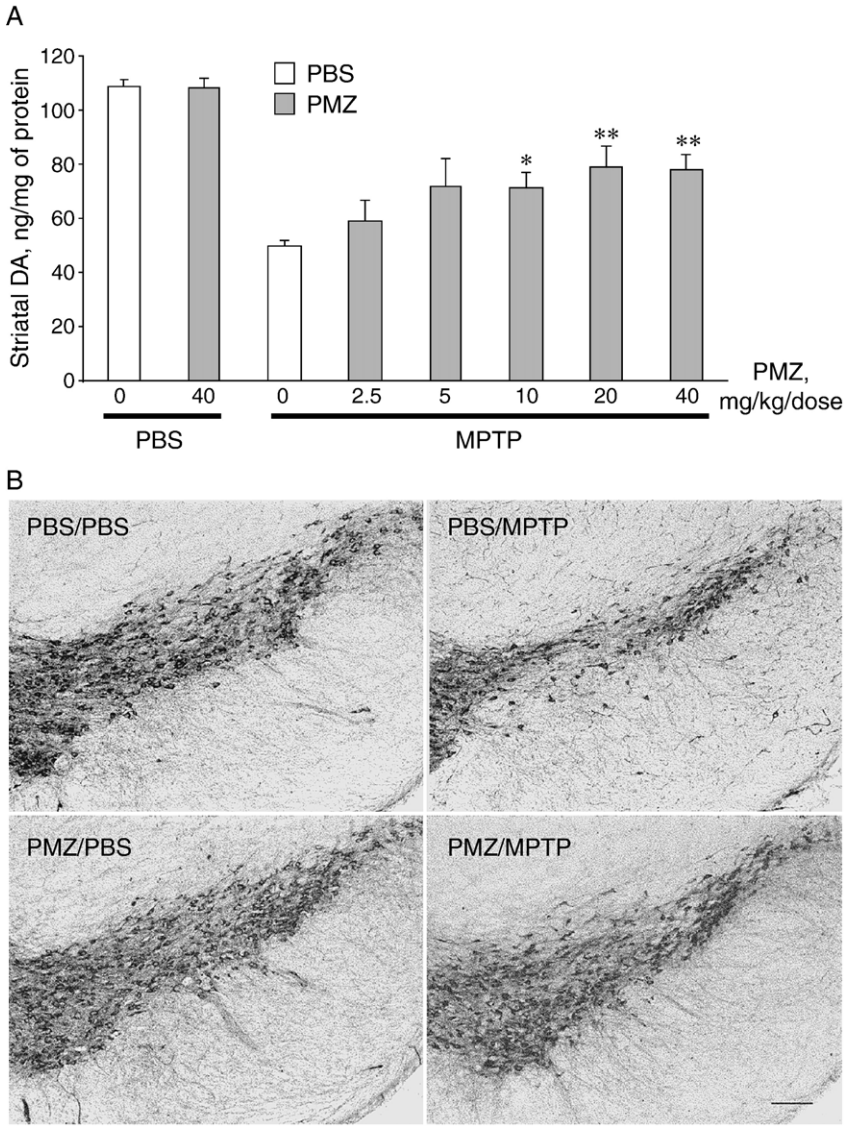

Fig. 1. Effects of PMZ treatment on striatal DA and nigral TH levels induced by MPTP treatment. (A) Injection of a 5 mg/kg/dose of PMZ, twice a day, was insufficient to protect against MPTP-induced decrease in DA levels, while the 10, 20, and 40 mg/kg/dose were protective. (B) TH immunoreactivity in the substantia nigra pars compacta of control or MPTP-lesioned mice treated with either PBS or PMZ. MPTP treatment (10 mg/kg/dose) produced a significant loss of TH-immunoreactive neurons in the substantia nigra pars compacta, while PMZ significantly mitigated the MPTP-induced loss of TH-positive neurons. Scale bar, 100 μm.

single MPTP injection (30 mg/kg ip). Mice were sacrificed 1, 2, 4, and 8 h later.

Protocol 3

MPTP-induced inhibition of complex I is an early and transient event (Sriram et al., 1997; Kenchappa and Ravindranath, 2003). Therefore, experiments involving measurements of complex I activity required us to sacrifice mice 15 min after the treatments. Mice (12–16 per group) were injected with PMZ (40 mg/kg ip) or PBS (10 ml/kg) 1 h before the first MPTP (10 mg/kg ip q 2 h × 4 doses) or PBS (10 ml/kg) injection. Mice were sacrificed 15 min after the last MPTP injection by cervical dislocation.

Mouse brain mitochondria were isolated by the Percoll gradient separation method as described (Sims, 1990) with minor modifications as follows. Animals were decapitated, the brain excised and placed into ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES–KOH (pH 7.4), 1 mM EGTA, and 1 g/l bovine serum albumin. The cerebellum was removed, and the rest of the brain tissue was placed in a 15-ml Dounce homogenizer and homogenized manually with 8 strokes of pestle A followed by 8 strokes of pestle B. The brain homogenate was centrifuged at 1250 × *g* for 3 min; pellet was discarded and the supernatant was centrifuged at 14,000 × *g* for 10 min. The pellet was resuspended in 15% Percoll (Sigma, St. Louis, MO, USA) and layered on a preformed Percoll gradient (40% and 23%). Following centrifugation at 30,000×*g* for 12 min, the mitochondrial fraction located at the interface of the lower two layers was removed, diluted with isolation buffer, and centrifuged at 14,000 × *g* for 10 min. The supernatant was discarded, and the loose pellet was resuspended in isolation buffer and centrifuged at 6700 × *g* for 10 min. The resulting pellet was suspended in 100 μl of isolation medium devoid of EGTA.

HPLC

For measurement of striatal dopamine or MPP^+ levels, dissected striata were immediately frozen on dry ice, and stored at −80°C. One striatum was sonicated and centrifuged in chilled 0.1 M perchloric acid (about 100 μl/mg tissue).

For measurements of dopamine, the supernatant was taken and analyzed by HPLC as modified from our previously described method (Beal et al., 1990). Ten microliters of supernatant was isocratically eluted through an 80 × 4.6 mm C18 column (ESA, Inc. Chelmsford, MA) with a mobile phase containing 75 mM of NaH_2PO_4, 1.5 mM OSA, 5% acetonitrile, pH 3, and detected by a two-channel Coulochem II electrochemical detector (ESA, Inc. Chelmsford, MA). The flow rate was 1 ml/min. Concentrations of dopamine were expressed as nanograms per milligram of protein. The protein concentrations of tissue homogenates were measured with the Bio-Rad protein analyze protocol (Bio-Rad Laboratories, Hercules, CA) and HTS7000+ plate reader (Perkin Elmer, Norwalk, CT).

For measurements of MPP^+, the supernatant was taken and analyzed by HPLC using a 4.6 mm × 250 mm Waters Xterra MSC18 5 μm column, Waters 515 pump, and a Waters 474 Scanning Fluorometer set at 295 nm excitation and 375 nm emission. The mobile phase was a pH 8.0, 20 mM borate buffer containing 3 mM tetrabutylammonium hydrogen sulfate and 0.25 mM 1-heptanesulfonic acid with 10% isopropanol (Naoi et al., 1987). Concentrations of MPP^+ are expressed as nanograms per milligram of wet tissue.

Histological and stereological analyses

Immunohistochemistry

Fresh brains were fixed by immersion in 4% paraformaldehyde (PFA) overnight at 4°C. Prior to sectioning, the tissues were placed in 30% glucose overnight at 4°C for cryoprotection. Serial coronal sections (50 μm thick) were cut through the substantia nigra or the striatum using a cryostat. Two sets consisting of eight sections each, 100 μm apart, were prepared. One set of sections was used for Nissl staining, and the other was processed for tyrosine hydroxylase (TH) immunohistochemistry using the avidin–biotin peroxidase technique (Vectastain ABC kit from Vector Labs, Burlingame, CA). A rabbit anti-TH affinity purified antibody (Chemicon, Temecula, CA; 1:3000) was used. The numbers of Nissl-stained or TH-immunoreactive cells in both sides of the substantia nigra pars compacta (SNpc) were estimated using the optical fractionator (West and Gundersen, 1990). Analysis was performed using a system consisting of a Nikon Eclipse E600 microscope equipped with a computer-controlled LEP BioPoint motorized stage, a DEI-750 video camera, a Dell Dimension 4300 computer, and the Stereo Investigator (v. 4.35) software program (Microbrightfield, Burlington, VT). Tissue sections were examined using a Nikon Plan Apo 100× objective lens with a 1.4 numerical aperture. The size of the x–y sampling grid was 140 μm. The counting frame thickness was 14 μm and the counting frame area was 4900 μm^2. The coefficient of error and coefficient of variation were also determined.

Enzyme assays

Complex I activity was measured spectrophotometrically as rotenone-sensitive NADH:Q_1 reductase. Reaction buffer was composed of 10 ml 2 mM HEPES (pH 7.8), 75 μM NADH, and 40 μM coenzyme Q_1 (final concentration). Frozen–thawed mice striata were homogenized in 2 mM HEPES buffer (pH 7.8) mixed with the reaction buffer in a 96-well plate and the absorbance changes at 340 nm were followed for 15 min with a plate reader HTS7000+ (Perkin Elmer, Norwalk, CT). In control incubations the reaction buffer was supplemented with 2 μM rotenone (final concentration). The activity of complex I was calculated as the difference between the rates of NADH oxidation (E^{340} mM = 6.22 cm^{-1}) in the absence and in the presence of rotenone and presented in nanomoles NADH per minute per milligram of wet tissue (Starkov and Fiskum, 2003).

Aconitase activity was measured spectrophotometrically by following the appearance of NADPH at 340 nm (Gardner, 2002). Reaction buffer was composed of 2 mM HEPES (pH 7.8), 0.6 mM $MnCl_2$, 0.5 mM $NADP^+$, 2 units of isocitrate dehydrogenase, 1 μM rotenone, 10 μM $CaCl_2$, and 1 mM citrate. Frozen–thawed rat cortex samples were homogenized in 2 mM HEPES buffer (pH 7.8) and mixed with the reaction buffer in a 96-well plate and the absorbance changes at 340 nm were followed for 15 min with a plate reader HTS7000+ (Perkin Elmer, Norwalk, CT).

Membrane potential ($\Delta\Psi$) of isolated mitochondria was estimated using the fluorescence of safranin O with excitation and emission wavelengths of 495 nm and 586 nm, respectively (Starkov et al., 2002). Incubation medium was composed of 125 mM KCl, 14 mM NaCl, 2 mM KH_2PO_4, 5 mM HEPES (pH 7.2), 4 mM $MgCl_2$, 3 mM ATP, 7 mM Pyruvate, 1 mM malate, and 2.5 μM safranin O, t = 37°C. Mouse brain mitochondria were added at 0.125 mg/ml, other

additions were as indicated in legends to figures. Fluorescence of safranin O was measured with an F4500 fluorimeter (Hitachi, Japan) equipped with a magnetic stirring assembly and a thermostated cuvette holder.

Mitochondrial permeability transition pore was assessed by monitoring the $Ca2^{+}$ retention and the swelling of mitochondria. Incubation medium was composed of 250 mM sucrose, 75 mM mannitol, 5 mM HEPES (pH 7.2), 2 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM EGTA, 0.2 μM Calcium Green 5N (calcium indicator), 10 mM succinate, and 1 μM rotenone, $t = 37°C$. Mouse brain or liver mitochondria were added at 0.25 mg/ml, other additions were as indicated in legends to figures. Changes in external Ca^{2+} were monitored as changes in Calcium Green 5N fluorescence measured at 506 nm excitation and 531 nm emission wavelengths with F4500 fluorimeter (Hitachi, Japan) equipped with a magnetic stirring assembly and a thermostated cuvette holder. Swelling of mitochondria was measured simultaneously as light scattering at 590/590 nm excitation/emission.

Statistical analysis

All data were computed in a database (Graphpad Instat® software) and expressed as mean ± SEM. Differences between groups and interaction between treatments were assessed by a Student's t test (unpaired) or by a one-way analysis of variance (ANOVA) followed, when appropriate, by a Student–Newman–Keuls post hoc test. A probability level of 5% ($P < 0.05$) was considered significant.

Results

Promethazine diminishes MPTP-induced neural tissue damage in mice

Mice that received MPTP (Protocol 1) sustained a 39% loss ($P < 0.001$) of Nissl-stained neurons (Table 1) and 37% ($P < 0.01$) loss of tyrosine hydroxylase (TH) immunopositive dopaminergic neurons within the SNpc, compared to sham-injected (PBS) mice (Table 1 and Fig. 1B). MPTP treatment decreased striatal dopamine levels by 43% ($P < 0.001$; Table 1).

Table 1
Effects of PMZ treatment on MPTP-induced neurotoxicity

	Nissl (average ± SEM)	TH (average ± SEM)	DA (average ± SEM)
PBS/PBS	12818.5 ± 678	9805.1 ± 616	103.0 ± 3
PMZ/PBS	12079.0 ± 384	9865.7 ± 522	97.4 ± 4
PBS/MPTP As compared to PBS/PBS	7758.6 ± 523, $P < 0.001$	6341.5 ± 777, $P < 0.01$	58.7 ± 6, $P < 0.001$
PMZ/MPTP As compared to PBS/MPTP	9960.0 ± 480, $P < 0.05$	8951.5 ± 649, $P < 0.01$	81.2 ± 5, $P < 0.01$

Total number of neurons in both sides of the substantia nigra pars compacta (SNpc) estimated after Nissl staining (cell body); total number of dopaminergic neurons in the SNpc counted after TH (tyrosine hydroxylase) immunohistochemistry; striatal level (ng/mg of protein) of dopamine (DA) measured by HPLC. Mice were sacrificed 7 days after treatments. PMZ protected against MPTP-induced depletion of these 3 parameters.

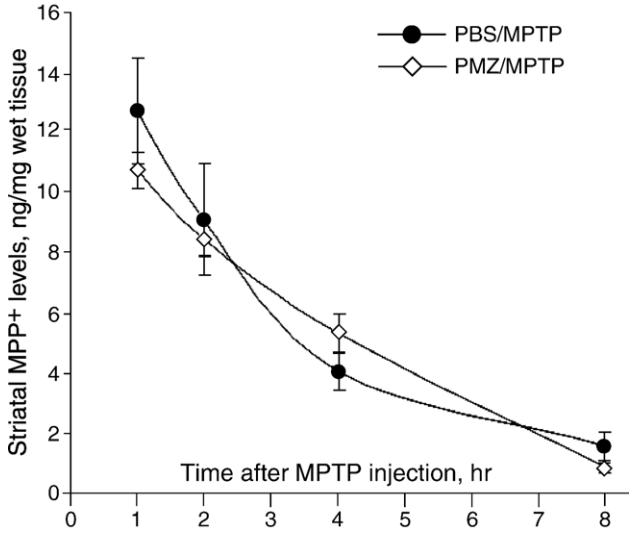

Fig. 2. Effects of PMZ pretreatment on striatal MPP^{+} levels induced by MPTP treatment. Mice were sacrificed 1, 2, 4, or 8 h after the first MPTP injection. PMZ treatment did not influence striatal MPP^{+} levels resulting from the MPTP injection. Striatal MPP^{+} levels diminished quickly and were nearly negligible 8 h after the injection.

Promethazine treatment (10 mg/kg ip q 12 h × 2 doses) significantly diminished the MPTP-induced loss of SNpc neurons. Indeed, PMZ protected Nissl-stained neurons ($P < 0.05$; Table 1) and TH-positive neurons ($P < 0.01$; Table 1 and Fig. 1B). PMZ also significantly protected against MPTP-induced depletion of striatal dopamine ($P < 0.01$; Table 1).

Promethazine does not affect the dynamic of MPP^{+} retention in mouse striata

The toxic metabolite of MPTP, MPP^{+}, accumulates in brain mitochondria (Ramsay and Singer, 1986) as does PMZ (Muller, 1996). Therefore, it was interesting to examine both the time dependence of MPP^{+} retention in mouse striata, and the effect of PMZ treatment on it. For these experiments, mice were treated with MPTP and PMZ (Protocol 2) and sacrificed 1, 2, 4, and 8 h after the MPTP injection. The striata were excised and frozen at −80°C. Frozen tissue was later thawed and used for measurements of MPP^{+} content and total aconitase activity. Fig. 2 shows that PMZ treatment did not affect MPP^{+} retention in mouse striata. Almost all MPP^{+} was gone from the tissue by 8 h. Both the initial concentrations of MPP^{+} in mouse tissue and the time course of its removal are in a good agreement with other studies utilizing similar MPTP treatment protocols (Liang and Patel, 2004).

Promethazine does not protect against MPTP-induced inhibition of mitochondrial complex I

MPTP is a well-known mitochondrial toxin targeting complex I (Nicklas et al., 1985) of the respiratory chain. We therefore examined the effect of PMZ treatment on mitochondrial enzyme activities in MPTP-treated mice.

MPTP inhibition of complex I in MPTP-treated mice was previously shown to be a transient event (Sriram et al., 1997; Kenchappa and Ravindranath, 2003). Therefore, we sacrificed animals 15 min after the MPTP injection (Protocol 3). This

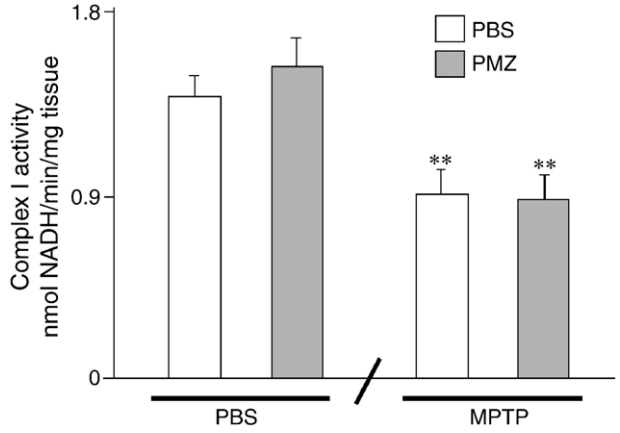

Fig. 3. Effect of PMZ treatment on MPTP-induced complex I inhibition in mice striatum. Mice were sacrificed 15 min after the last MPTP injection. $^{**}P < 0.001$ as compared to PBS/PBS-treated rats.

protocol yielded a significant inhibition of complex I activity in mice striata. PMZ treatment did not prevent complex I inhibition (Fig. 3).

Promethazine prevents MPTP-induced transient inhibition of aconitase in mouse striatum

Aconitase activity is very sensitive to inhibition by superoxide and therefore considered to be a good in vivo tissue marker of oxidative stress (Gardner, 2002). MPTP treatment (Protocol 2) resulted in a transient inhibition of total aconitase activity in mouse striata 2 h after MPTP injection ($P < 0.05$), which was significantly reversed by the PMZ treatment ($P < 0.05$). However, since the 2-h activity in PBS/MPTP-treated mice is similar to the 4-h activity of PMZ/MPTP-treated mice, we cannot exclude the hypothesis that PMZ treatment may only delay MPTP-induced depression in aconitase activity (Fig. 4).

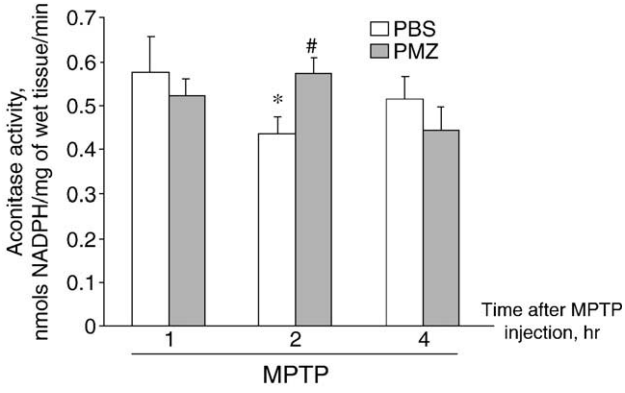

Fig. 4. Effects of PMZ treatment on MPTP-induced inhibition of aconitase activity. Mice were sacrificed 1, 2, or 4 h after the first MPTP injection. Two hours after MPTP treatment aconitase activity was transiently inhibited. PMZ treatment prevented the MPTP-induced aconitase inhibition. $^{*}P < 0.05$ as compared to PBS/MPTP-treated rats 1 h after the MPTP injection; $^{\#}P < 0.05$ as compared to PBS/MPTP-treated rats 2 h after the MPTP injection.

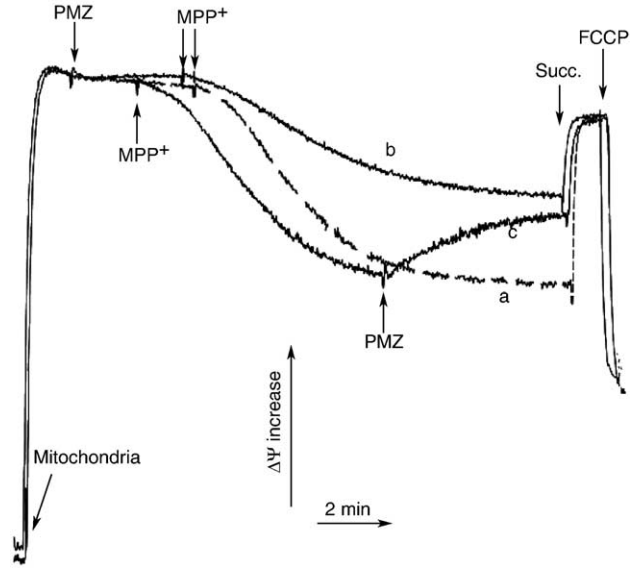

Fig. 5. Effect of PMZ and MPP^+ additions on mitochondrial membrane potential. Isolated brain mitochondria were incubated in a cytosolic-like medium: PMZ (125 μM) both attenuated (curve b) and reversed (curve c) MPP^+ (200 μM)-induced mitochondrial membrane depolarization (curve a).

Promethazine both attenuated and reversed MPP^+-induced mitochondrial membrane depolarization in vitro

The toxicity of MPTP in vivo is thought to be at least partially due to the inhibitory effect of its metabolite, MPP^+, on mitochondrial complex I (Nicklas et al., 1985). It is well established that in vitro, MPP^+ inhibits the respiration of brain mitochondria supported by a complex I-related NAD^+-linked substrates such as pyruvate and malate (Mizuno et al., 1988); however, the effect of MPP^+ on the mitochondrial membrane potential has not yet been reported. We examined both the effect of MPP^+ and PMZ on the membrane potential and the respiration of

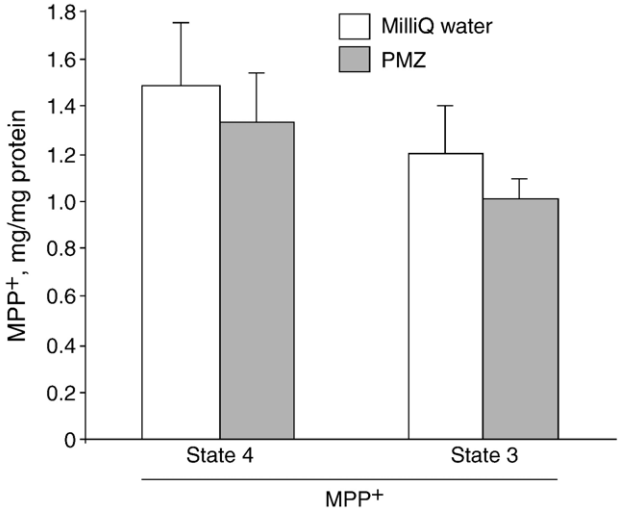

Fig. 6. Effect of PMZ on MPP^+ accumulation in mitochondria during active and resting states. Brain mitochondria were incubated with MPP^+ (200 μM) in the presence or in the absence of PMZ (125 μM). PMZ did not affect MPP^+ accumulation by mitochondria in either resting state (state 4) or active (phosphorylating: state 3).

mouse brain mitochondria. Isolated mitochondria were incubated in a cytosol-like high ionic strength medium supplemented with pyruvate and malate. The addition of 200 μM MPP^+ induced a slow depolarization of mitochondria (Fig. 5, curve a). The slow dynamic of MPP^+-induced depolarization of mitochondria is most likely due to the slow penetration of MPP^+ through the mitochondrial membrane to its target site in the complex I (Ramsay and Singer, 1986). The addition of PMZ had no effect on the membrane potential. PMZ added in the medium either before or after MPP^+, partially attenuated (Fig. 5, curve b) and reversed (Fig. 5, curve c) respectively MPP^+-induced mitochondrial membrane depolarization. The addition of succinate to MPP^+-depolarized mitochondria restored the membrane potential, thereby indicating that the decrease was indeed due to the inhibition of complex I or NAD^+-linked primary substrate dehydrogenases, and not due to an uncoupling or membrane damage induced by MPP^+ accumulation.

As described above, PMZ alone had no effect on the mitochondrial membrane potential. However, it was possible that PMZ, which can be accumulated in mitochondria (Muller, 1996), interfered with MPP^+ accumulation, thereby decreasing the effective MPP^+ concentration in mitochondria. Therefore, we measured MPP^+ accumulation in mitochondria. For this purpose, mitochondria were incubated with 200 μM MPP^+ in the absence or in the presence of 125 μM PMZ. When present, PMZ was added 2 min before MPP^+. PMZ did not affect MPP^+ accumulation by mitochondria in either active (phosphorylating) or resting states (Fig. 6).

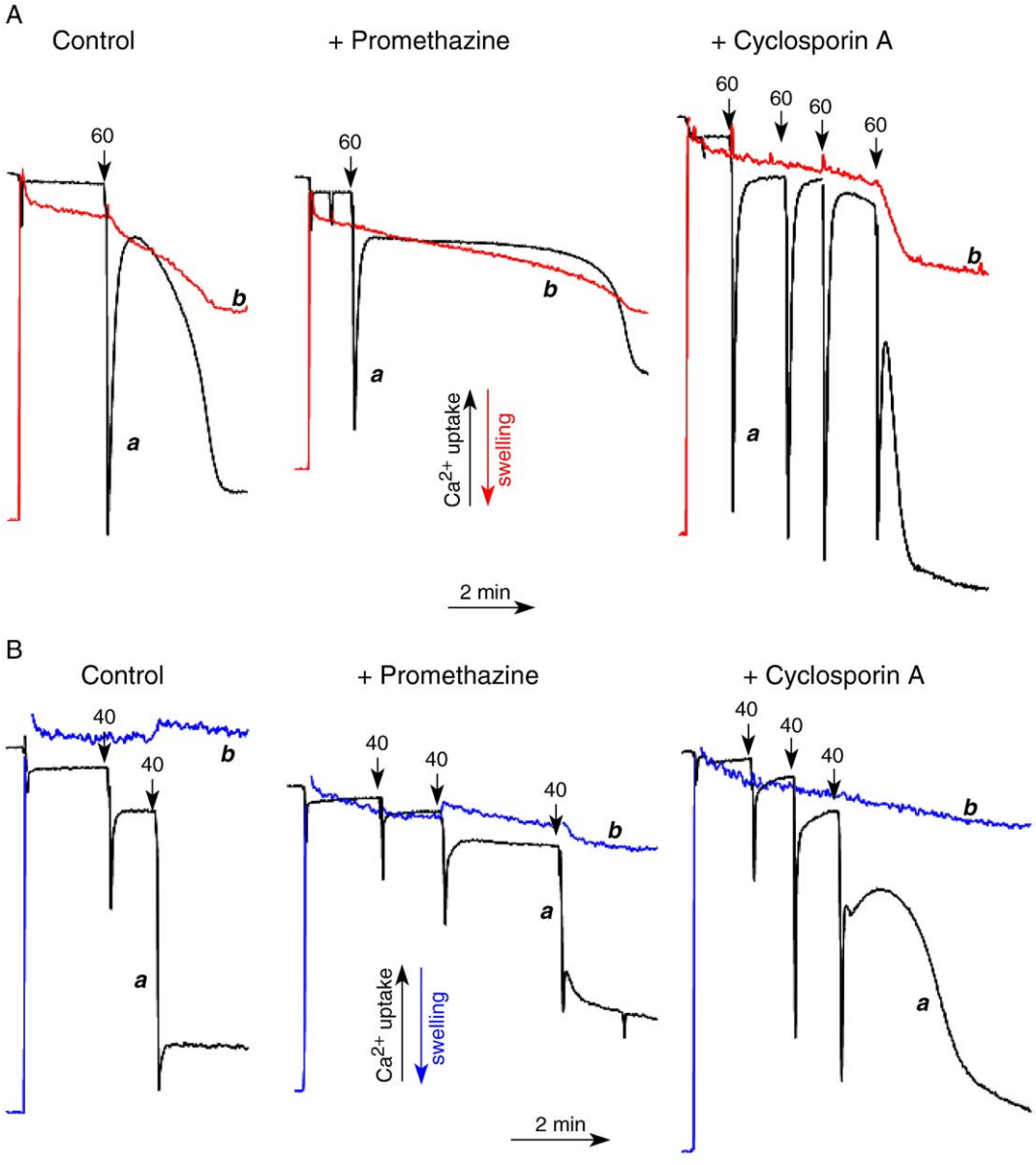

Fig. 7. Effect of PMZ on mitochondrial calcium capacity. (A) Isolated liver mitochondria were incubated in a mannitol/sucrose medium. PMZ (10 μM) increased the calcium capacity threshold for the mitochondrial permeability transition pore (middle panel) to a similar extent as cyclosporin A (0.5 μM, right panel). Sixty nanomoles of Ca^{2+} was added per milligram of mitochondria. (B) Isolated brain mitochondria were incubated in a mannitol/sucrose medium. PMZ (10 μM) increased the calcium capacity threshold for the mitochondrial permeability transition pore (middle panel) in a similar extent as cyclosporin A (0.5 μM, right panel). Forty nanomoles of Ca^{2+} was added per milligram of mitochondria.

PMZ increased the Ca^{2+} threshold for the induction of mitochondrial permeability transition

Earlier, PMZ was shown to delay the Ca^{2+}-induced mitochondrial permeability transition pore in rat liver mitochondria (Stavrovskaya et al., 2004). We found that 10 μM PMZ significantly extended (~3.5 times) the lag period between the Ca^{2+} loading and the activation of the permeability transition pore in mouse liver mitochondria (Fig. 7A). The opening of the PTP was manifested as a spontaneous progressing release of accumulated Ca^{2+} (Fig. 7A, left panel, curve a) accompanied by the swelling of liver mitochondria (Fig. 7A, left panel, curve b). In the presence of promethazine, the spontaneous release of Ca^{2+} was significantly delayed (Fig. 7A, middle panel, curve a) and the swelling of mitochondria was less pronounced (Fig. 7A, middle panel, curve b). In liver mitochondria, cyclosporin A significantly elevated the Ca^{2+} threshold for the induction of the PTP (Fig. 7A, right panel, curves a).

Essentially similar effects were observed in experiments with isolated mouse brain mitochondria incubated under the same conditions. Promethazine increased the Ca^{2+} retention and the Ca^{2+} threshold for the induction of PTP (Fig. 7B, middle panel) with an efficiency similar to that of cyclosporin A (Fig. 7B, right panel). Ca^{2+} loading of mouse brain mitochondria under our experimental conditions did not induce swelling. This is in agreement with earlier published reports addressing the differences in response of rodent brain and liver mitochondria on Ca^{2+} challenge and PTP inhibitors, showing that brain mitochondria do not swell as liver mitochondria do (Andreyev and Fiskum, 1999; Berman et al., 2000).

Discussion

The data presented in this manuscript allow us to conclude that PMZ is a strong neuroprotective compound capable of attenuating neurodegeneration induced by MPTP in mice. Indeed, PMZ prevented both the neurodegeneration of nigrostriatal dopaminergic neurons and the loss of striatal dopamine induced by MPTP in mice. In addition, we showed that PMZ protected isolated mouse brain mitochondria against MPP^+-induced depolarization of the membrane potential and against the Ca^{2+}-induced permeability transition.

Inhibition of complex I of the mitochondrial respiratory chain by MPTP metabolite, MPP^+, is thought to play a significant role in the molecular mechanism of MPTP-induced neurotoxicity (Przedborski and Jackson-Lewis, 1998). It is therefore quite interesting that inhibition of complex I in striatal tissue of mice treated with MPTP resolved as early as 2 h after the last MPTP injection, which is in agreement with previous publications (Sriram et al., 1997; Kenchappa and Ravindranath, 2003). However, the concentration of MPP^+ in striatal tissue at this time point was still high (Fig. 2) and also in agreement with values published earlier (Sriram et al., 1997). The transient character of MPTP-induced inhibition of complex I in striatal tissue has not yet been fully explained.

PMZ treatment clearly protected brain tissue against MPTP-induced toxicity but did not rescue the activity of complex I (Fig. 3), neither did it change the retention of MPP^+ in striatal tissue (Fig. 2). However, PMZ treatment protected the loss of aconitase activity seen 2 h after MPTP treatment (Fig. 4). Aconitase activity is thought to be a marker of oxidative stress because the enzyme is inhibited by superoxide (Gardner, 2002). Therefore, our data indicate that PMZ may protect against MPTP-induced oxidative stress.

PMZ was capable of rescuing the mitochondrial membrane potential of isolated mouse brain mitochondria that was decreased by the addition of MPP^+ (Fig. 5). In these experiments, 200 μM MPP^+ induced a slow decrease in the membrane potential of mitochondria incubated under either non-phosphorylating conditions ("resting state", in the absence of ADP; Fig. 5, curve a) or phosphorylating conditions (data not presented). PMZ did not change the accumulation of MPP^+ in brain mitochondria (Fig. 6); however, it partially attenuated (Fig. 5, curve b) and/or restored (Fig. 5, curve c) the membrane potential decrease induced by MPP^+ (Fig. 5, curve a). The effect of PMZ was dose dependent, with the strongest effect at the 125-μM concentration, shown in Fig. 5. At the concentrations used in our experiments, PMZ had no effect on the membrane potential, in agreement with an earlier study using lower concentrations of the drug (Stavrovskaya et al., 2004). The molecular mechanism of this PMZ effect is under investigation; our preliminary data indicate that PMZ affects the electron transfer reactions within complex I (Starkov et al., 2004).

Low concentrations of PMZ increased the threshold level of mitochondrial Ca^{2+} accumulation needed to induce the permeability transition pore in mouse liver (Fig. 7A) and brain mitochondria (Fig. 7B). This effect of PMZ was reported earlier with rat liver mitochondria and was thought to be responsible for tissue protection against damage induced by ischemia (Narayanan et al., 2004; Stavrovskaya et al., 2004). We confirmed the observations of these authors and extended it to both mouse liver and brain mitochondria.

The mechanism of the protective action of PMZ requires further research. In this manuscript, we describe a novel direct protective effect of PMZ on mitochondrial membrane potential and show that it inhibits activation of the PTP in brain mitochondria. The neuroprotection we observed suggests that PMZ could play a role in preventing and/or delaying the onset of PD.

Acknowledgments

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